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Atypical phenotype and response of B cells in patients with seropositive rheumatoid arthritis

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Summary

Patients with rheumatoid arthritis (RA) may be classified as seropositive or seronegative according to the presence of autoantibodies. An abnormal B cell phenotype and function could be one of the main components of the immunopathology of seropositive patients; however, there is little information regarding B cell defects in these patients. This study shows a broad characterization of the B cell phenotype and function in patients with seropositive RA. We focused mainly on the evaluation of subsets, the expression of modulatory molecules of cell activation (CD22, FcγRIIb and FcμR), calcium mobilization, global tyrosine phosphorylation, expression of activation markers, cytokine and immunoglobulin (Ig) production, proliferation and the *in-vitro* generation of plasma cells. Increased frequency of $CD27^{-}IgM^{-}IgD^{-}$ and $CD21^{-}$ B cells was observed in patients with seropositive RA compared with healthy donors (HD). Decreased expression of CD22 was primarily found in memory B cells of patients with RA regardless of seropositivity. B cells from seropositive patients exhibited normal proliferation, calcium mobilization kinetics and global tyrosine phosphorylation, but showed an increased frequency of $CD86^{+}$ B cells compared with HD. B cells of seropositive patients secrete less interleukin-10 after *in-vitro* activation and showed a decreased frequency of plasma cell differentiation and IgM production compared with HD. Our data indicate that patients with seropositive RA have an increased frequency of atypical B cell populations previously associated with chronic activation and antigen exposure. This may result in the observed low responsiveness of these cells *in vitro*.

Keywords: B cell, B cell activation, B cell memory, B cell subsets, CD21, CD22, exhaustion, plasma cell, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease with a diverse clinical presentation primarily characterized by the chronic inflammation of synovial tissue. This can cause irreversible damage to the joints if not properly treated [1]. Patients with RA can be classified as seropositive or seronegative based on the presence of hallmark autoantibodies. Anti-cyclic citrullinated protein antibodies (ACPAs) and rheumatoid factor (RF) are the primary autoantibodies associated with seropositive RA [2]. ACPAs are useful for diagnosis and play an important role in the pathogenesis of RA [3]. The presence of ACPAs is considered a poor prognostic factor in disease development [4]. Their presence is directly associated with radiographic severity [5], erosive disease [6], systemic inflammation [7] and worse physician global assessment ratings in RA patients [8]. ACPAs induce the secretion of cytokines, such as tumor necrosis factor (TNF)-α and receptor activator of nuclear factor kappa-B ligand (RANKL), by directly binding to citrullinated components on mononuclear phagocytes or indirectly through the formation of immune complexes (ICs). This promotes the differentiation and activation of osteoclast and bone erosion [9–12]. In addition, RF can bind to ICs formed by the ACPAs resulting in an increase in the size and

complexity of these structures and the response of the innate immune system [13].

It has been reported that seropositive patients respond more favorably to rituximab, a biological compound that targets and depletes $CD20^+$ B cells, compared with seronegative patients [14]. The efficacy of both rituximab and anti-TNF-α therapies in seropositive patients has been associated with a significant reduction in ACPA levels [15,16]. Therefore, it has been suggested that the abnormal B cell phenotype and function must be one of the main components in the immunopathology of seropositive RA patients [1]. High levels of ACPA in the serum of patients with seropositive RA were positively correlated with the frequency of ACPA-producing class-switched memory B cells [$CD27^+$immunoglobulin (Ig)D^-] [17]. ACPA-expressing B cells from seropositive patients exhibit a memory cell phenotype and express IgG or IgA [18]. In addition, B cells may participate in the immunopathogenesis of seropositive RA by altering the production of both proinflammatory [interleukin (IL)-6 and TNF-α] and anti-inflammatory (IL-10) cytokines, as well as in their antigen-presenting and co-stimulatory activities [19].

A variety of molecules can negatively regulate B cell responses, including FcγRIIb and CD22. FcγRIIb (CD32b) is an inhibitory Fc receptor that binds to IgG ICs and regulates B cell responses [20]. FcγRIIb deficiency results in increased disease severity in a murine model of collagen-induced arthritis [21]. In humans, some FcγRIIb polymorphisms have been associated with susceptibility to systemic lupus erythematosus (SLE) [22]. FcμR is the most recent Fc receptor described in B cells. Interestingly, mice deficient in FcμR are more prone to produce autoantibodies [23], suggesting that this receptor controls the development of autoimmunity. CD22 is an inhibitory receptor that belongs to the lectin family and down-regulates B cell receptor (BCR)/Toll-like receptor (TLR) signaling in B cells [24]. B cells from $CD22^{-/-}$ mice exhibit increased plasma cell differentiation and the production of antibodies after primary immunization with proteins coupled to haptens [25]. Also, decreased expression of CD22 on B cells has been described in patients with active lupus [26] and systemic sclerosis [27]. However, clinical trials evaluating agonist antibodies against this molecule (epratuzumab) have not yielded promising results in the treatment of lupus [28]. To our knowledge, the expression of these receptors has not been studied in patients with seropositive RA. Considering the importance of FcγRIIb, FcμR and CD22 in B cell responses and their association with autoimmunity, alterations in these molecules in patients with seropositive RA may contribute to the pathogenesis of this disease.

We hypothesize that B cells from patients with seropositive RA have a phenotype that is associated with functional alterations in these cells. In this study, we evaluated the expression of CD22, FcγRIIb and FcμR on B cell subsets from patients with seropositive RA. In addition, different functional and general responses of these cells in patients with seropositive RA, including calcium mobilization, global tyrosine phosphorylation, expression of activation markers, proliferation and generation of plasma cells, were evaluated.

Materials and methods

Patients and controls

Twenty-three patients with long-onset RA, diagnosed according to the American College of Rheumatology and the European League Against Rheumatism [29] criteria, were recruited from 2016 to 2018 at the Rheumatology Unit, Hospital Universitario San Vicente Fundación (HUSVF, Medellin, Colombia). Eleven healthy donors (HD) with the same gender and similar ages (≤ 2 years) as the patients were also included in this study. Patients were classified into two groups according to positivity for anti-cyclic citrullinated peptide-3 (CCP3) IgG, without including IgA or other autoantibodies or isotypes: seronegative ($CCP3^-$) and seropositive ($CCP3^+$). Most patients were under treatment (drugs summarized in Table 1); none of them received biological therapy. Patients were also classified according to Disease Activity Score 28 (DAS28) using C reactive protein for remission (DAS28 ≤ 2·6, rRA) or active (DAS28 > 2·6, aRA) disease. The main demographic and clinical data for the patients and HD were obtained from medical records and personal interviews (Table 1). All participants signed an informed consent

Table 1. Demographic and clinical characteristics of patients with rheumatoid arthritis (RA)

		Patients with RA	
	HD	Seronegative	Seropositive
n	11	6	17
Age[a]	53 (38–67)	55.5 (41–66)	56 (39–69)
Female	11/11	6/6	16/17
DAS28[a]	n.a.	1.90 (1.25–5.22)	2.65 (1.60–5.93)
CCP3 IgG U/ml[a]	n.a.	7.98 (6.78–9.86)	911.14 (45.48–2838.86)
RF-IgM U/ml[a]	n.a.	8.69 (0–11.47)	58.97 (1.62–20.51)
Therapies			
Methotrexate	n.a.	0/6	8/17
Prednisolone	n.a.	3/6	7/17
Leflunomide	n.a.	1/6	6/17
Chloroquine	n.a.	1/6	4/17
Deflazacort	n.a.	0/6	2/17
Sulfasalazine	n.a.	2/6	1/17

[a]Median (range). HD = healthy donor; n.a. = not applicable; DAS28 = disease activity score 28; RF-IgM = rheumatoid factor-immunoglobulin M; CCP3 = anti-cyclic citrullinated peptide 3.

previously approved by the ethics committee from Instituto de Investigaciones Médicas (Facultad de Medicina, Universidad de Antioquia, Medellin, Colombia) and HUSVF.

Isolation of mononuclear cells and enrichment of B cells

Fresh peripheral blood mononuclear cells (PBMCs) were isolated from ethylenediamine tetraacetic acid (EDTA)-anti-coagulated blood collected from HD and RA patients by centrifugation at 900 ***g*** for 30 min at room temperature using Histopaque (Sigma-Aldrich, St Louis, MO, USA). Cell suspensions were washed twice with phosphate-buffered saline (PBS; Gibco, Carlsbad, MA, USA) at 250 ***g*** for 10 min. The total cell number and viability (> 97%) were calculated using a Neubauer chamber and trypan blue exclusion (Sigma-Aldrich), respectively. The reported number of cells was calculated based on PBMC counts and frequency of $CD19^+$ cells and B cell subsets as detected by flow cytometry. These data were normalized to the total amount of blood (ml) used to isolate PBMCs for each individual. B cells were enriched (80–90% purity) from fresh EDTA anti-coagulated blood using rosettes (Stem Cell Technologies, Vancouver, BC, Canada), following the manufacturer's instructions. In all cases, PBMCs and B cells were processed immediately after isolation.

Antibodies

Anti-human CD10-allophycocyanin/cyanin 7 (APC/Cy7) (clone HI10a), CD19-brilliant violet (BV) 650 (clone HIB19), CD22-BV711 (clone S-HCL-1), CD24-BV605 (clone ML5), CD27-phycoerythrin (PE)/Cy7 (clone O323), CD38-BV785 (clone HIT2), CD69-BV711 (clone FN50), CD80-PE (clone 2D10), CD86-BV510 (clone IT2.2), CD95-Pacific blue (clone DX2), CD138-APC/Cy7 (clone MI15), IgD-AlexaFluor700 (clone IA6-2) and IgM-PE/CF594 (clone MHM-88) monoclonal antibodies were purchased from Biolegend (San Diego, CA, USA). Anti-human monoclonal antibody against FcγRIIb-eFluor450 (clone 6C4) was obtained from eBiosciences (San Jose, CA, USA) and FcμR-PE (clone HM7-10) was purchased from Becton Dickinson (San Diego, CA, USA).

B cell subsets and phenotype

PBMCs were incubated with blocking buffer [10% fetal bovine serum (FBS, Gibco), 0·1% bovine serum albumin (Sigma-Aldrich) and 0·01% sodium azide (Sigma-Aldrich) in PBS, pH = 7·35] for 15 min at 4°C. PBMCs were labeled together with anti-human CD10, CD19, CD21, CD24, CD27, CD38, IgM, IgD, FcμR, FcγRIIb and CD22 in PBS and incubated for 30 min at 4°C in the dark. Cells were then washed and immediately analyzed using an LSRFortessa flow cytometer with FACSDIVA software (Becton Dickinson). For MitoTracker staining (deep red; Thermo-Fisher Scientific, Waltham, MA, USA), PBMCs were incubated with 250 pM of the dye in RPMI-1640 media (Gibco) for 15 min at 37°C. Cells were washed with PBS and stained with the antibody mix as described above. The results are presented as relative and absolute counts or mean fluorescence intensity (MFI).

Calcium mobilization

A total of 1×10^5 B cells from HD and patients with seropositive and inactive RA (in remission, rRA) were incubated with 2 μg/ml Indo-1 AM calcium dye (Thermo-Fisher Scientific) and 0·045% Pluronic F-127 (Invitrogen, Carlsbad, CA, USA) in complete medium [RPMI-1640 Glutamax, 10% FBS, 2 mM L-glutamine and 1% streptomycin/penicillin (Gibco)] for 15 min, as previously described [30]. The cells were washed with RPMI-1640 and stained with anti-human CD19, CD21, CD24, CD27, CD38 and IgM monoclonal antibodies for 15 min at room temperature. Then, the cells were washed and resuspended in 450 μl of complete medium and maintained at 37°C for 10 min. Immediately, a baseline acquisition was obtained during the first 30 s by flow cytometry. B cells were stimulated with 10 μg/ml polyclonal anti-human IgM/IgG $F(ab)_2$ fraction (Jackson Immunoresearch, West Grove, PA, USA). Each sample was analyzed for a total of 120 s at 37°C. The relative concentration of intracellular free calcium was calculated according to the ratio of calcium-bound and calcium-free (unbound) as detected by the changes of Indo-1 MFI at 405/485 nm, respectively.

Global tyrosine phosphorylation

B cells from HD and patients with seropositive rRA were suspended at 1×10^6 cells/ml in serum-free medium. After 10 min incubation at 37°C, the B cells were unstimulated or stimulated with 10 μg/ml polyclonal anti-human IgM $F(ab)_2$ fraction (Jackson ImmunoResearch) for 45 s. Sodium pervanadate was used as a positive control for phosphorylation [31]. Thereafter, the cells were kept on ice for 15 min and labeled with anti-human CD19, CD21, CD24, CD27, CD38 and IgM monoclonal antibodies. Cells were fixed and permeabilized using the forkhead box protein 3 (FoxP3)/transcription factor staining buffer set kit (eBiosciences) for 1 h following the manufacturer's instructions, as previously described for phosphoproteins [32]. Cells were then incubated with anti-phosphotyrosine antibody (clone PY20, eFluor450; eBiosciences) for 30 min in 100 μl of permeabilization buffer. Finally, the cells were washed with permeabilization buffer and PBS, and immediately analyzed by flow cytometry. Because each subpopulation of B cells exhibits different basal levels of global

tyrosine phosphorylation, the results are presented as fold-change of phosphorylation, where the condition of unstimulated B cell subsets was used to normalize the respective data. Fluorescence minus one (FMO) was used as the staining control to determine the positive events.

Activation markers, cytokine production and proliferation

Enriched B cells from HD and patients with seropositive rRA were cultured in complete medium with 10 ng/ml IL-2 (Biolegend) at 2×10^6 cells/ml. To evaluate the expression of activation markers on B cells and cytokine production, the cells were unstimulated or stimulated with 2·5 μg/ml anti-human IgM/IgG $F(ab)_2$ fraction alone or in combination with 2·5 μg/ml cytosine–phosphate–guanine oligodeoxynucleotide (CpG) (Invivogen, Carlsbad, CA, USA) for 24 and 72 h [33]. Supernatants were collected and stored at −20°C until cytokine measurement. To evaluate proliferation, enriched B cells were stained with CellTrace Violet Cell Proliferation reagent (Thermo-Fisher) before culture, following the manufacturer's instructions. Then, the cells were stimulated with the same concentrations of anti-IgM/IgG $F(ab)_2$ fraction plus CpG for 72 h. To determine the expression of activation markers and proliferation, the cells were stained with Live/Dead fixable blue dead cell stain kit (Live/Dead; Thermo-Fisher), following the manufacturer's instructions. After a blocking step, the cells were immediately labeled with anti-human CD69, CD80, CD86, CD19, CD21, CD24, CD27, CD38, IgD and IgM and immediately analyzed by flow cytometry.

Plasma cell differentiation and antibody production

Enriched B cells from HD and patients with seropositive rRA (2×10^6 cells/ml) were cultured for 7 days in complete medium with 10 ng/ml IL-2 under two different conditions: (1) 50 ng/ml IL-21 (Biolegend) plus 1 μg/ml CD40L (R&D Systems, Minneapolis, MN, USA) and (2) 2·5 μg/ml anti-IgM/IgG $F(ab)_2$ fraction plus 50 ng/ml IL-21 and 1 μg/mL CD40L [33,34]. Supernatants were collected and stored at −20°C until the total immunoglobulin and autoantibody measurements. Cells were stained with Live/Dead following the manufacturer's instructions. After a blocking step, cells were labeled with anti-human CD19, CD21, CD24, CD27, CD38 and IgM monoclonal antibodies and analyzed by flow cytometry. The results are presented as frequencies.

Cytokines, autoantibodies and total IgG and IgM

IL-8, IL-6, IL-10 and TNF-α levels were measured in the supernatants of cultured B cells using the human inflammatory cytometric bead array (Becton-Dickinson), following the manufacturer's instructions, and analyzed by flow cytometry. IgM and IgG levels were measured in supernatants of cultured B cells using an enzyme-linked immunosorbent assay (ELISA) Ready-Set-Go! kit (eBiosciences). RF-IgM and ACPA IgG were measured in patients' sera and in supernatants of cultured B cells using Quanta-Lite RF-IgM and Quanta-Lite CCP3-IgG ELISA kits (< 20 U/ml was considered a negative result; Innova Diagnostics, San Diego, CA, USA), all according to the manufacturer's instructions. Measurements were made using an ELx800 absorbance microplate reader (Biotek Instruments, Winooski, VT, USA) at 450 nm emission wavelength.

Data analysis

Flow cytometry data [gating analyses, MFI, proliferation (division index and percentage of divided cells) and subset frequencies] were analyzed using the FlowJo software version X package (Tree Star, San Carlos, CA, USA). T-distributed stochastic neighbor embedding (t-SNE) analysis was performed using FlowJo software after gating single cells and lymphocyte regions. The concatenated file consisted of 90 000 events in the lymphocyte region from each donor, and were down-sampled from five HD and five patients with seropositive RA. To generate a representative t-SNE plot, a perplexity of 30 and an iteration number of 1000 were used. The frequencies of B cells and MFI data between HD and patients with seropositive and seronegative RA were compared with the Kruskal–Wallis test and Dunn's post-test. Data for cultured B cells from HD and patients were analyzed with a two-way analysis of variance (ANOVA) test and a Šidák post-test. Statistical analyses were performed using Prism version 6.0 software (GraphPad, San Diego, CA, USA) and P-values < 0·05 were considered statistically significant. Comparisons were conducted among all the study groups; however, only those that were statistically significant are shown in the figures.

Results

Increased frequency of $CD27^-IgM^-$ and $CD21^-$ B cells in patients with seropositive RA

To characterize whether there were changes in total B cells from patients with RA compared with HD with respect to seropositivity ($CCP3^+$ and $CCP3^-$), $CD19^+$ PBMC were analyzed (Fig. 1a). No differences in frequency or number of total B cells were observed in the study groups (Fig. 1b). PBMCs from five HD and five patients with seropositive RA were labeled with anti-human CD19, CD10, CD21, CD24, CD27, CD38, IgM and IgD antibodies and analyzed using the t-SNE algorithm. As expected, B cells were primarily enriched in two clusters based on the expression of the BCR (Fig. 1c). B cells from patients with seropositive RA and HD shared a similar t-SNE distribution (Fig. 1c). However, comparison of the B cell markers used in this analysis between patients with seropositive RA and HD revealed global differences in the

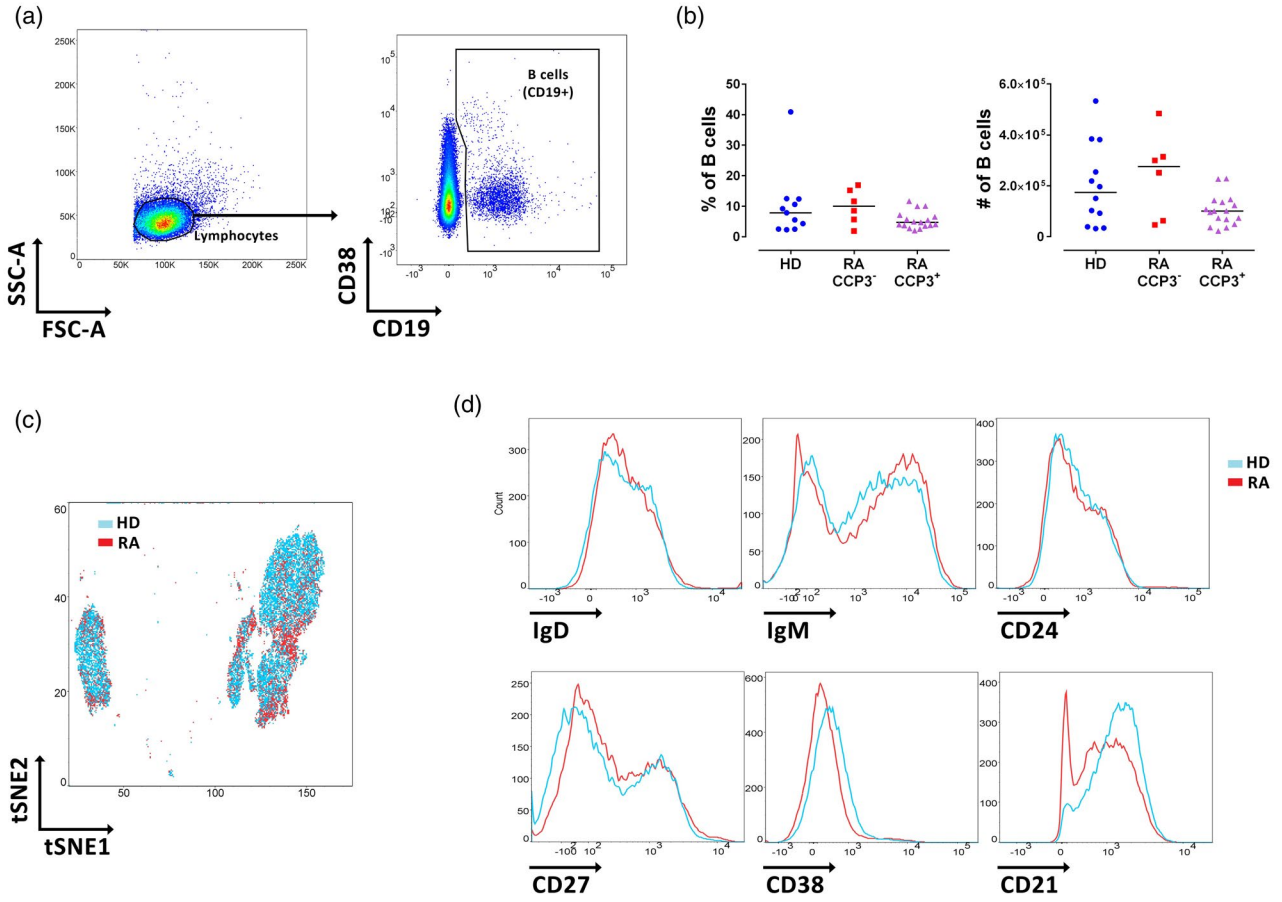

Fig. 1. B cells from patients with seropositive rheumatoid arthritis (RA) exhibit a different pattern of marker expression used to define subsets. (a) Representative pseudocolor plots showing the data analysis of CD19$^+$ B cells from a healthy donor (HD) after aggregate exclusion (not shown), selection of lymphocytes and exclusion of death cells according to forward-scatter (FSC)-A and side-scatter (SSC)-A parameters. (b) Frequency and number of total CD19 cells from HD and patients with seronegative (CCP3$^-$) and seropositive (CCP3$^+$) RA according to the analysis shown in (a). Data and median of 11 HD, six patients with CCP3$^-$ RA and 17 patients with CCP3$^+$ RA are shown. Kruskal–Wallis test and Dunn's post-test. (c) t-SNE analysis was performed with concatenation of the same number of lymphocytes from five HD and five patients with seropositive RA. Overlay of t-SNE maps of CD19$^+$ cells from HD (blue) and patients with seropositive RA (red). (d) Histogram overlay of B cell markers used for t-SNE alaysis from HD (blue) and patients with seropositive RA (red).

expression of different molecules, particularly IgM, CD27, CD38 and CD21 (Fig. 1d). The results suggested that patients with seropositive RA may have alterations in a particular subgroup of B cells rather than a general defect in these cells.

To further explore this finding, a detailed analysis of B cell subsets was performed. According to the relative expression of CD24 and CD38, total B cells were further divided into transitional (CD24^{hi}CD38^{hi}) and plasma (CD24$^-$CD38^{hi}) cells (Fig. 2a). There were no significant changes in the frequency of these B cell subsets in patients with RA (Fig. 2a). CD38$^{low/-}$ B cells were subsequently gated according to CD27 expression. Once again, no differences in the frequency of total CD27$^+$ (memory) and CD27$^-$ B cells were observed among the study groups (Fig. 2a). As the t-SNE analysis (Fig. 1c,d) revealed differences in the expression of IgM, CD38$^{low/-}$ cells were then classified into CD27$^-$IgM$^+$, CD27$^+$IgM$^+$, CD27$^+$IgM$^-$ and CD27$^-$IgM$^-$ subsets. Interestingly, this approach allowed us to detect an increased frequency, but not number, of CD27$^-$IgM$^-$ B cells in patients with seropositive RA (CCP3$^+$) compared with HD and seronegative patients (CCP3$^-$) (Fig. 2b and Supporting information, Fig. S1). There were no changes in the frequency of B cell subsets when patients were studied with respect to disease activity (Supporting information, Fig. S2).

Finally, the frequency of CD21$^-$ cells in the B cell population was evaluated. Patients with seropositive RA exhibited a higher frequency of CD21$^-$ cells compared with HD (Fig. 2c). CD21$^-$ cells were negative for CD10 in all donors (Fig. 2d), suggesting that they do not correspond to immature B cells. These data show that patients

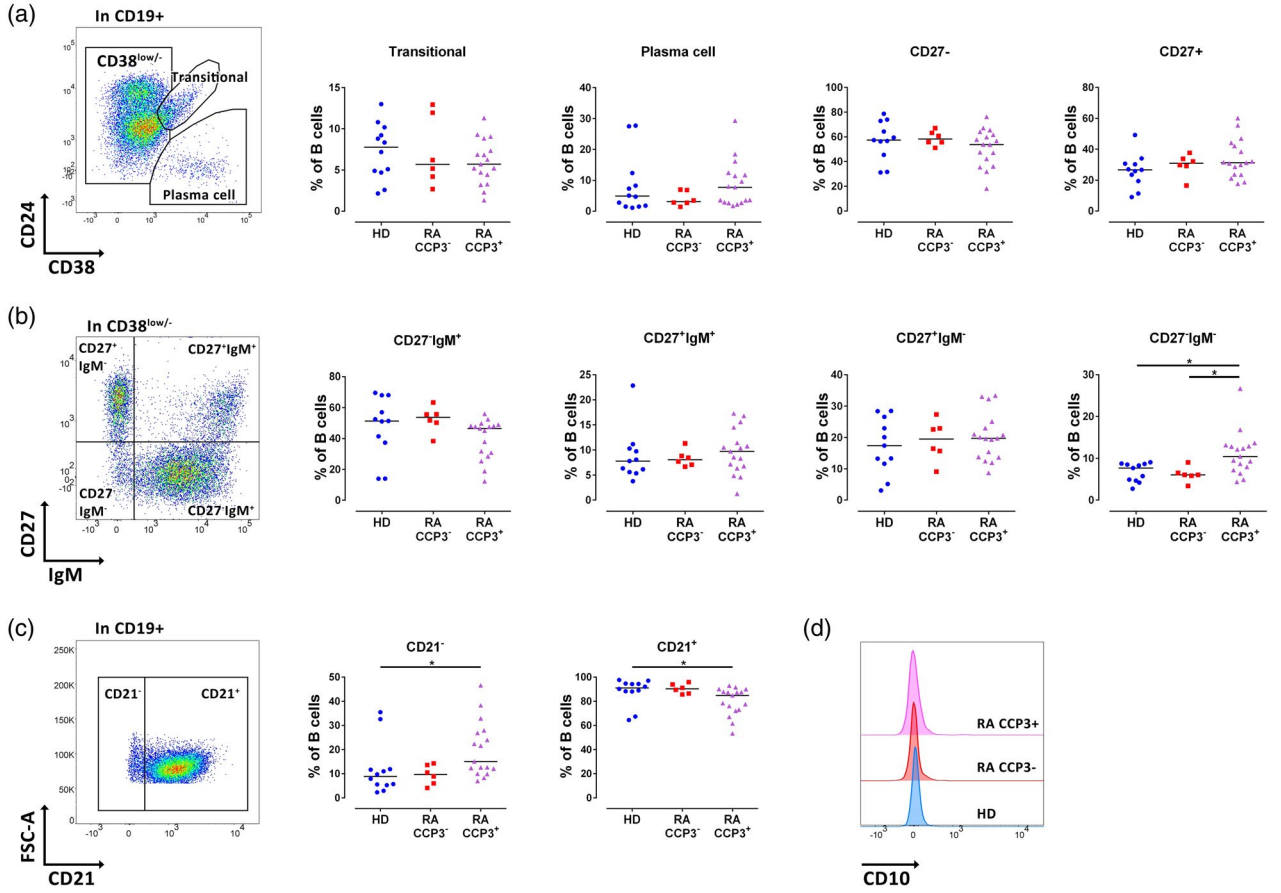

Fig. 2. Increased frequency of CD27$^-$immunoglobulin (Ig)M$^-$ and CD21$^-$ B cells in patients with seropositive rheumatoid arthritis (RA). (a) Representative pseudocolor plot (left) showing the data analysis of plasma, transitional and CD38$^{low/-}$ B cell subsets from a HD. Frequency of transitional, plasma cell, CD27$^-$ and CD27$^+$ subsets from HD and patients with seronegative (CCP3$^-$) and seropositive (CCP3$^+$) RA. (b) Representative pseudocolor plot (left) from a HD showing the data analysis of CD38$^{low/-}$ cells according to CD27 and IgM relative expression. Frequency of CD27$^-$IgM$^+$, CD27$^+$IgM$^+$, CD27$^+$IgM$^-$ and CD27$^-$IgM$^-$ subsets in HD and patients with CCP3$^-$ and CCP3$^+$ RA. (c) Representative pseudocolor plot showing the analysis of CD19$^+$ cells according to CD21 expression from a HD. Frequency of CD21$^-$ and CD21$^+$ B cells from HD and patients with CCP3$^-$ and CCP3$^+$ RA. (d) Representative histograms of CD10 expression on CD21$^-$ cells from HD and patients with CCP3$^-$ and CCP3$^+$ RA. Data and median of 12 HD, six patients with CCP3$^-$ RA and 17 patients with CCP3$^+$ RA are shown. Kruskal–Wallis test and Dunn's post-test, $^*P < 0{\cdot}05$.

with seropositive RA have an increased frequency of CD27$^-$IgM$^-$ and CD21$^-$ B cells.

Increased frequency of atypical activated/memory B cells in patients with seropositive RA

To further evaluate the alterations observed in B cell subsets from patients with seropositive RA, CD27$^-$IgM$^-$ and CD21$^-$ cells were characterized in more detail. As some CD27$^-$IgM$^-$ B cells are anergic (IgD$^+$) [35], we evaluated both CD27$^-$IgM$^-$IgD$^+$ and CD27$^-$IgM$^-$IgD$^-$ populations. An increased frequency of CD27$^-$IgM$^-$IgD$^-$ B cells was observed in patients with seropositive RA, but no significant differences were evident in cells with an anergic phenotype (Fig. 3a). CD27$^-$IgM$^-$IgD$^-$ B cells exhibited a pattern of MitoTracker signal more similar to activated/memory B cells (CD27$^+$IgM$^-$) compared with naive (CD27$^-$IgM$^+$) B cells in patients with seropositive RA (Fig. 3b). Next, the expression of CD21 was evaluated in CD27$^-$IgM$^-$IgD$^-$ B cells. Increased frequency of CD21$^-$ cells in CD27$^-$IgM$^-$IgD$^-$ population was observed in patients with seropositive RA compared to HD (Fig. 3c). These data suggest that the increased frequency of the atypical CD27$^-$IgM$^-$IgD$^-$ B cells observed in seropositive patients could be related to activated/memory phenotype and the increase of CD21$^-$ B cells.

CD21$^-$ B cells were classified into transitional (CD38^{hi}CD24^{hi}), plasma cells (CD38^{hi}CD24$^-$) and CD38$^{low/-}$ cells (Fig. 3d). Then, CD21$^-$CD38$^{low/-}$ B cells were analyzed according to CD24 and CD27 expression (Fig. 3d) or IgM and CD27 expression (Fig. 3e) to

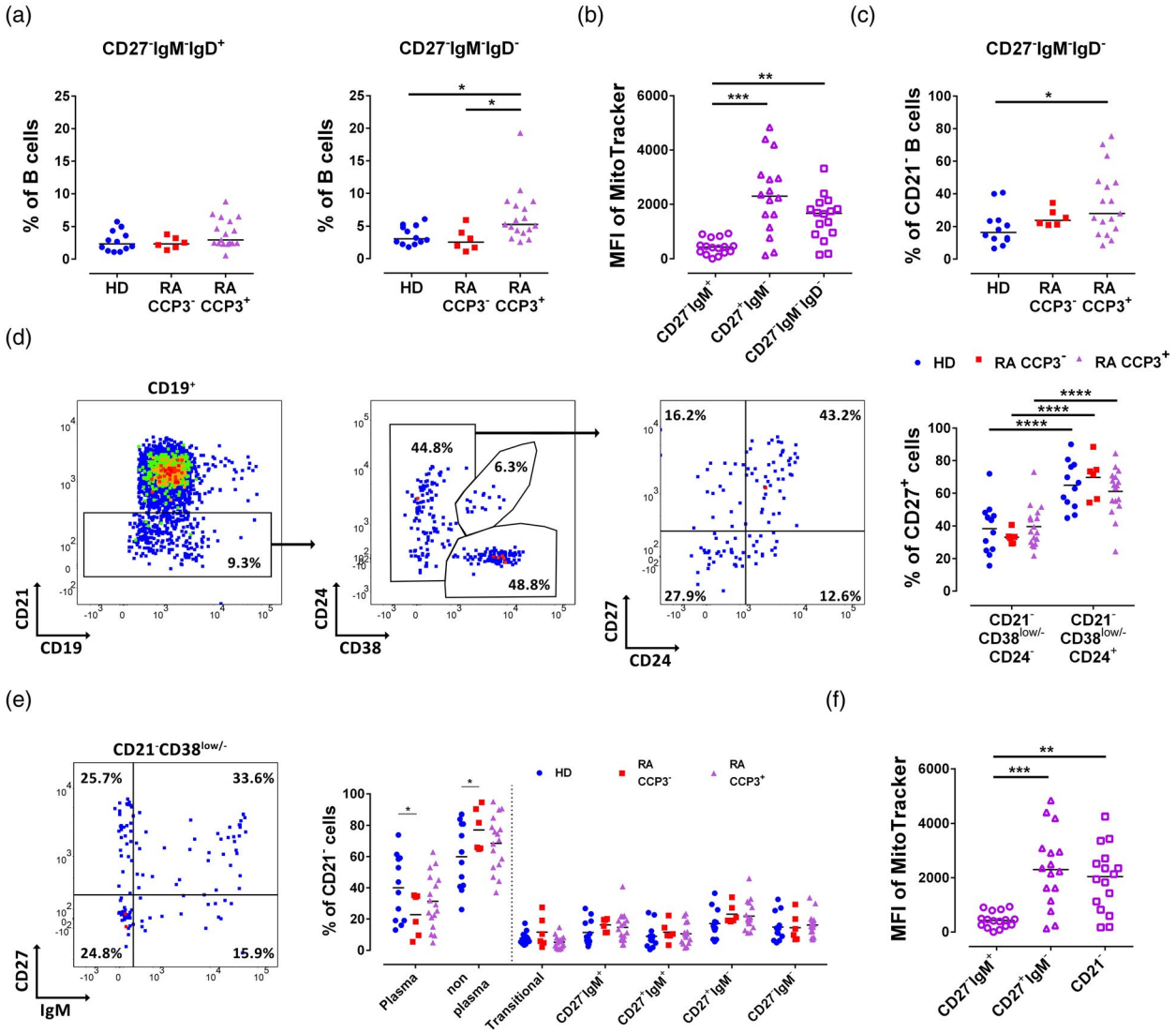

Fig. 3. Increased frequency of atypical CD27⁻IgM⁻IgD⁻ and CD21⁻ B cells in patients with seropositive rheumatoid arthritis (RA). (a) Frequencies of CD27⁻immunoglobulin (IgM)⁻IgD⁺ and CD27⁻IgM⁻IgD⁻ B cells. (b) Mean fluorescence intensity (MFI) of MitoTracker in CD27⁻IgM⁺, CD27⁺IgM⁻ and CD27⁻IgM⁻IgD⁻ B cells. Median and data from 16 patients with CCP3⁺ RA are shown. (c) Median and frequencies of CD21⁻ cells in CD27⁻IgM⁻IgD⁻ B cells of 12 healthy donors (HD), six patients with CCP3⁻ RA and 17 patients with CCP3⁺ RA are shown. Kruskal–Wallis test, Dunn's post-test. (d) Analysis of CD21⁻ B cells into plasma cells (CD38⁺CD24⁻), transitional (CD38⁺CD24⁺) and CD38$^{low/-}$ cells. Then, CD21⁻CD38$^{low/-}$ B cells were divided according to CD24 and CD27 expression. Comparison of frequencies (right) of CD27⁺cells in CD21⁻CD38$^{low/-}$CD24⁺ and CD21⁻CD38$^{low/-}$CD24⁻B cells. (e) CD21⁻CD38$^{low/-}$ B cells were analyzed according to IgM and CD27 expression (CD27⁻IgM⁺, CD27⁺IgM⁺, CD27⁺IgM⁻ and CD27⁻IgM⁻), and a comparison of these subpopulation frequencies as well as plasma cells and transitional B cells among the study groups is shown. (d,e) Mean and frequencies of 12 HD, six patients with CCP3⁻ RA and 17 patients with CCP3⁺ RA are shown. Two-way analysis of variance (ANOVA) test with Šidák post-test. (f) MFI of MitoTracker of CD27⁻IgM⁺, CD27⁺IgM⁻ and CD21⁻ B cells. Median and data from 16 patients with CCP3⁺ RA are shown. Kruskal–Wallis test with Dunn's post-test. (a–e) *$P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$, **** $P < 0{\cdot}0001$.

characterize their memory phenotype. In general, approximately 60 and 50% of the total CD21⁻CD38$^{low/-}$ cells express CD27 and CD24, respectively. No differences in frequency of CD27⁺ cells in CD21⁻CD38$^{low/-}$CD24⁻ and CD21⁻CD38$^{low/-}$CD24⁺ B cells were observed among the study groups. However, CD21⁻CD38$^{low/-}$CD24⁺ B cells had a higher frequency of CD27⁺ cells compared to CD21⁻CD38$^{low/-}$CD24⁻ B cells in all study groups (Fig. 3d). To further evaluate the potential enrichment of memory phenotype in CD21⁻ B cells, these cells were classified into six subsets (plasma/plasmablast, transitional, CD27⁻IgM⁺, CD27⁺IgM⁺, CD27⁺IgM⁻ and CD27⁻IgM⁻ B cells). Decreased frequency of plasma cells and increased frequency of non-plasma cells in CD21⁻ cells were observed

in patients with seronegative RA compared with HD (Fig. 3e). No differences in $CD21^-$ B cells with transitional, $CD27^-IgM^+$, $CD27^+IgM^+$, $CD27^+IgM^-$ and $CD27^-IgM^-$ B cell phenotypes were observed among study groups (Fig. 3e). MitoTracker signal has been previously reported as increased in memory B cell populations [36]. MitoTracker signal evaluated in total $CD21^-$ cells [and in the different subsets: $CD21^-CD38^+CD24^-$ ($CD21^-$ plasma cells), $CD21^-CD38^{low/-}$, $CD21^-CD38^{low/-}CD24^-$ and $CD21^-CD38^{low/-}CD24^+$ B cells] were more similar to memory ($CD27^+IgM^-$) B cells than the naive ($CD27^-IgM^+$) subset (Fig. 3f and data not shown).

These results suggest that the expansion of atypical $CD21^-$ and $CD27^-IgM^-IgD^-$ B cells in patients with seropositive RA did not localize into a classical B cell subset and seem more closely related to activated/memory B cells [37] than anergic or naive B cells.

Decreased expression of CD22 on memory B cell subsets from patients with RA irrespective of seropositivity

Because FcγRIIb, FcμR and CD22 molecules can modulate B cell activation [38,39], the expression of these receptors was evaluated in each B cell subset from patients with seronegative and seropositive RA (Supporting information, Fig. S3A). A lower expression of CD22 was observed in transitional ($CD24^{hi}CD38^{hi}$) and naive (IgM^+CD27^-) B cells of patients with seronegative RA compared with HD (Fig. 4a). Also, lower expression of CD22 was observed in total $CD27^+$ B cells from patients with both seronegative and seropositive RA compared with HD (Fig. 4a). This reduced CD22 expression was observed in all memory subsets ($CD27^+IgM^+$, $CD27^+IgM^-$ and $CD27^-IgM^-$) from patients with seronegative and seropositive RA (Fig. 4a). A similarly decreased expression of CD22 was observed in patients independent of disease activity (Fig. 4b). No differences in FcμR or FcγRIIb expression were observed in B cell subsets from patients with RA that were classified with respect to seropositivity or DAS28 score (Supporting information, Fig. S3). These results indicate that patients with RA exhibit lower expression of CD22 in different B cell subsets, but primarily in memory B cells, regardless of seropositivity or disease activity.

B cells from patients with seropositive RA have a normal calcium mobilization kinetics and global tyrosine phosphorylation

To evaluate the activation and functional response of B cells in patients with seropositive RA, we examined calcium mobilization and global tyrosine phosphorylation in these cells. No significant differences in the kinetics of calcium mobilization were observed in patients with seropositive RA compared with HD (Fig. 5a). As expected, the area under the curve gradually increased after anti-IgM/IgG stimulation, but no significant differences in these values were observed between HD and patients (Fig. 5b). Similarly, no differences in calcium mobilization were found when B cell subsets were compared between patients with RA and HD (Fig. 5c). We also evaluated calcium mobilization in B cells with anergic phenotypes that were previously described by others as $CD27^-CD21^-$ [40] and $CD27^-IgM^-IgD^+$ [35]. No significant differences were evident in calcium mobilization in these two populations between HD and patients with seropositive RA (Supporting information, Fig. S4).

Anti-IgM stimulation increased the MFI of global tyrosine phosphorylation (p-Tyr) of B cells (Fig. 5d,e). No significant differences in MFI of p-Tyr in total B cells (Fig. 5e) or the subsets (Fig. 5f) were observed in seropositive patients compared with HD. These data indicate that B cells do not appear to have alterations in early transduction of signals through the BCR in patients with seropositive RA.

Increased expression of CD86 and low secretion of cytokines in B cells from patients with seropositive RA

To further characterize B cell responses in patients with seropositive RA, these lymphocytes were stimulated with anti-BCR plus CpG for longer periods to evaluate the expression of activation markers, proliferation and cytokine production. As anticipated, the activation of B cells increased the frequency of $CD69^+$, $CD80^+$ and $CD86^+$ cells in both HD and patients with RA (Fig. 6a,b). However, a higher frequency of $CD86^+$ B cells was observed in patients compared with HD when the cells were treated with anti-IgM/IgG for 24 h and anti-BCR plus CpG for 72 h (Fig. 6b). This significant increase in CD86 expression was mainly detected in $CD21^-$ cells at 24 h of culture after anti-BCR plus CpG stimulus (data not shown).

Increased frequency of proliferating cells and the index of division were observed in B cells from patients and HD treated with anti-BCR plus CpG for 72 h compared with unstimulated cultures. However, no differences between patients and HD were observed (Fig. 7a). The majority of proliferating B cells were $CD27^-$, both in HD and patients. No differences were observed in frequency of $CD27^+$ or $CD27^-$ proliferating B cells between HD and patients (Fig. 7b). Higher IL-6 and IL-10 levels after 24 h (Fig. 7c) and higher IL-10 and TNF-α levels after 72 h of culture (Fig. 7d) were detected in the supernatants of anti-BCR plus CpG-stimulated B cells from HD compared with unstimulated cells. Nevertheless, B cells from patients with RA did not show a significant increase in these cytokines compared with HD (Fig. 7d). No production of these cytokines was observed for anti-IgM/IgG treatment alone in either study group (data not shown).

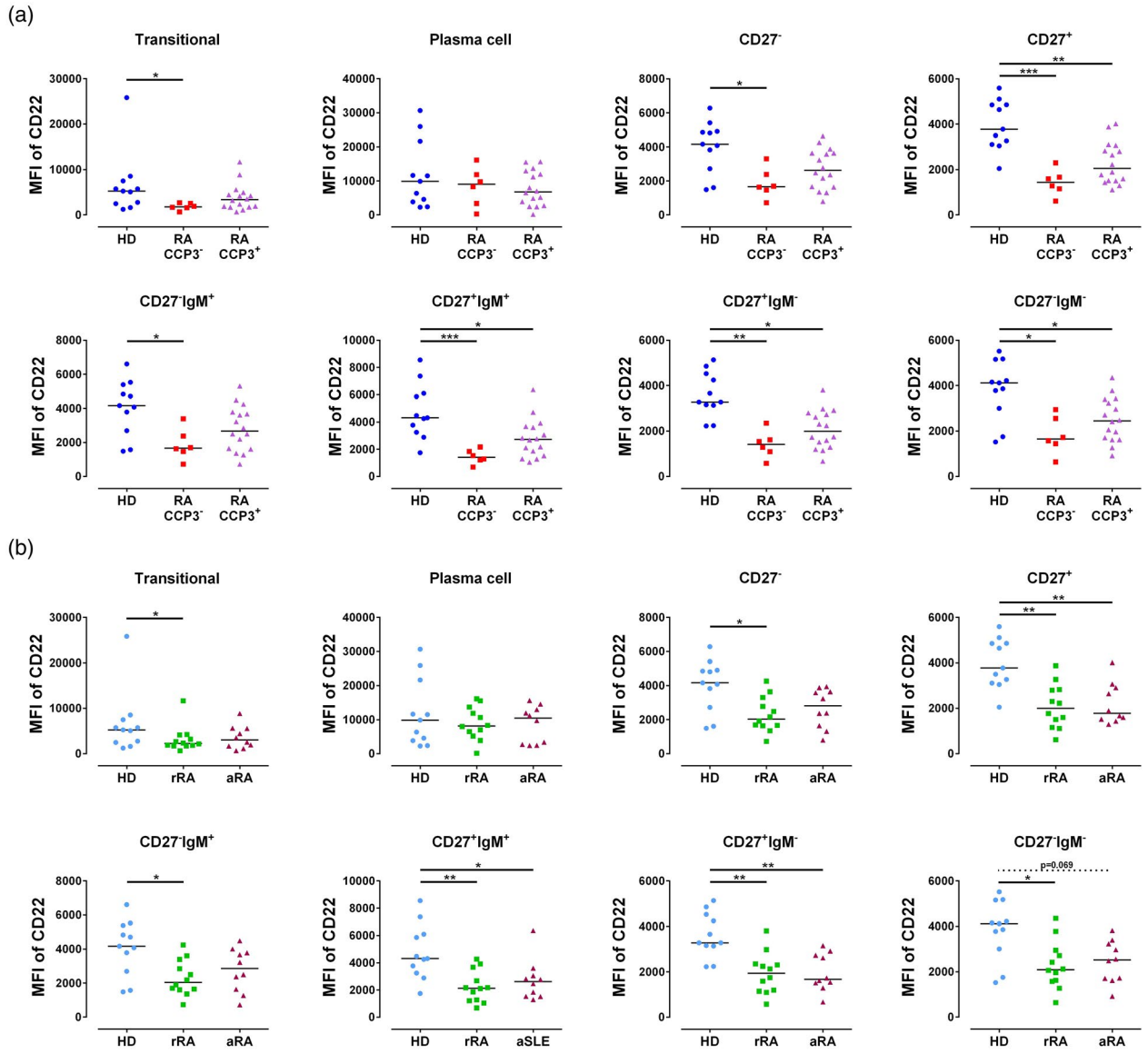

Fig. 4. Decreased expression of CD22 on B cells from patients with rheumatoid arthritis (RA). Comparison of the mean fluorescence intensity (MFI) of CD22 in B cell subsets between healthy donors (HD) and patients with RA according to (a) autoantibody presence, seropositive ($CCP3^+$) and seronegative ($CCP3^-$); and (b) disease activity score 28 (DAS28), patients with active (DAS28 > 2·6, aRA) disease and in remission (DAS28 ≤ 2·6, rRA). Data and median of 10 HD, six patients with $CCP3^-$ RA, 16 patients with $CCP3^+$ RA, 12 rRA patients and 10 aRA patients are shown. Kruskal–Wallis test, Dunn's post-test, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

These data show that B cells from seropositive patients exhibit a high frequency of $CD86^+$ cells and low secretion of cytokines following *in-vitro* activation.

Decreased *in-vitro* generation of plasma cells in patients with seropositive RA

The final outcome of B cell activation is the generation of memory and plasma cells. To address this, enriched B cells from HD and patients with seropositive RA were stimulated with CD40L and IL-21 in the presence or absence of anti-IgM/IgG for 7 days. An increased frequency of $CD19^+CD38^{low/-}$ B cells and decreased plasma cells ($CD19^{low}CD38^+$) were observed in patients compared with HD after CD40L plus IL-21 treatment (Fig. 8a,b). To understand if the increase of $CD19^+CD38^{low/-}$ cells corresponds to a particular subset, the viability and switched status was evaluated according to CD27 and IgM expression. No differences were observed in the frequency of live cells or $CD27^-IgM^+$, $CD27^+IgM^+$, $CD27^+IgM^-$ and $CD27^-IgM-$ subsets in $CD19^+CD38^{low/-}$ cells in either group (Supporting information, Fig. S5 and data not shown). Similarly, no

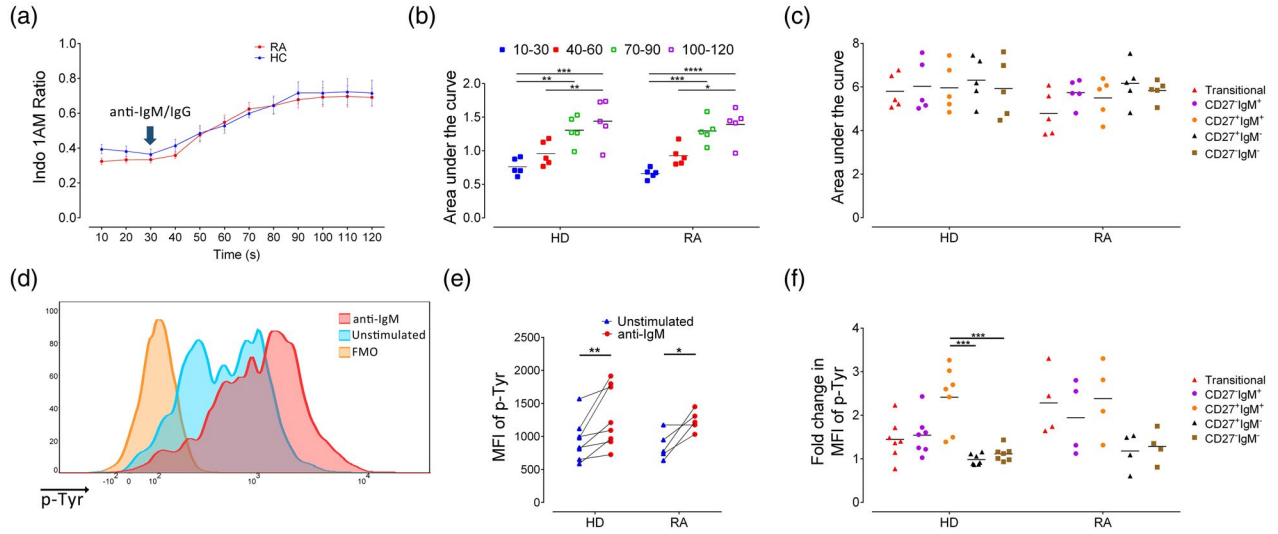

Fig. 5. No differences in calcium mobilization and global phosphorylation of tyrosine between patients with seropositive rheumatoid arthritis (RA) and healthy donors (HD). (a) Kinetic of Indo 1AM ratio in total B cells unstimulated for 30 s (baseline) and stimulated with anti-immunoglobulin (Ig) M/IgG for 90 s. (b) Area under the curve of the data shown in (a) for time intervals of 10–30, 40–60, 70–90 and 100–120 s. (c) Area under the curve of the data shown in (a) for each B cell subset. (a–c) Mean and data of five HD and five patients with seropositive RA are shown. Standard error of the mean is also shown in (a). (b) Representative histograms of global tyrosine phosphorylation (p-Tyr) in total B cells of an HD: FMO (orange), unstimulated (blue) and stimulated cells with anti-IgM (red) for 45 s. (e) Mean fluorescence intensity (MFI) of p-Tyr in total B cells before (unstimulated) and after activation (anti-IgM) in eight HD and five patients with seropositive RA according to the analysis shown in (d). Wilcoxon test. (f) Fold change of p-Tyr MFI of each B cell subset. (e,f) Data and mean from seven HD and five patients with RA are shown. (a–c, f) Two-way analysis of variance (ANOVA) test with Šidák post-test. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, $^{****}P < 0{\cdot}0001$.

differences in total IgG, anti-CCP3 IgG or RF-IgM were detected in the supernatants of activated B cells in either group (Fig. 8c and data not shown). Decreased production of total IgM was observed in enriched B cells from patients after CD40L plus IL-21 treatment compared with HD (Fig. 8c). These data suggest that B cells from patients with seropositive RA tend to differentiate less toward plasma cells *in vitro* compared with HD.

Discussion

In the present study, we performed a general characterization of both the phenotype and function of B cells from patients with seropositive RA. We found that B cells from patients with seropositive RA exhibited a higher frequency of CD21^{-} and CD27^{-}IgM^{-}IgD^{-} B cells, lower CD22 expression, augmented frequency of CD86^{+} cells and decreased differentiation of plasma cells *in vitro*.

A higher frequency in CD27^{-}IgD^{-} B cells has been previously described in patients with RA compared with HD [41]. Our results are consistent with this report, and also further demonstrate that the increased proportion of CD27^{-}IgM^{-}IgD^{-} B cells primarily occurs in patients with seropositive RA. The memory CD27^{-} subset is composed of class-switched cells that exhibit high levels of somatic hypermutations [41]. These cells have also been observed in a high proportion of patients with other autoimmune diseases, including SLE [42] and multiple sclerosis [43]. Therefore, the increase of these cells in a patient with seropositive RA may indicate a more constant antigenic exposure, for example to citrullinated peptides, compared with seronegative patients.

Similarly, we observed an increased frequency of CD21^{-} B cells. The higher frequency of the plasma cells in the CD21^{-} B cell population compared with other subsets was an expected finding, because the expression of CD21 is down-regulated in this cellular subset [44]. However, plasma cells were under-represented in the CD21^{-} cells from patients; it is possible that this cell subset in patients could be already differentiated into long-lived plasma cells that are mainly located in bone marrow or, alternatively, part of these cells could remain in the tertiary lymphoid structures associated with local tissue inflammation [45]. The non-plasma CD21^{-} B cells have been described in HD as anergic [40] or memory [46] cells. In our study, these cells were more closely related to the memory phenotype but did not localize into a particular B cell subset. A high frequency of CD21^{-} B cell-expressing autoreactive BCR was previously described in RA [40]. In the present report, we also showed that an increase of CD21^{-} cells is characteristic

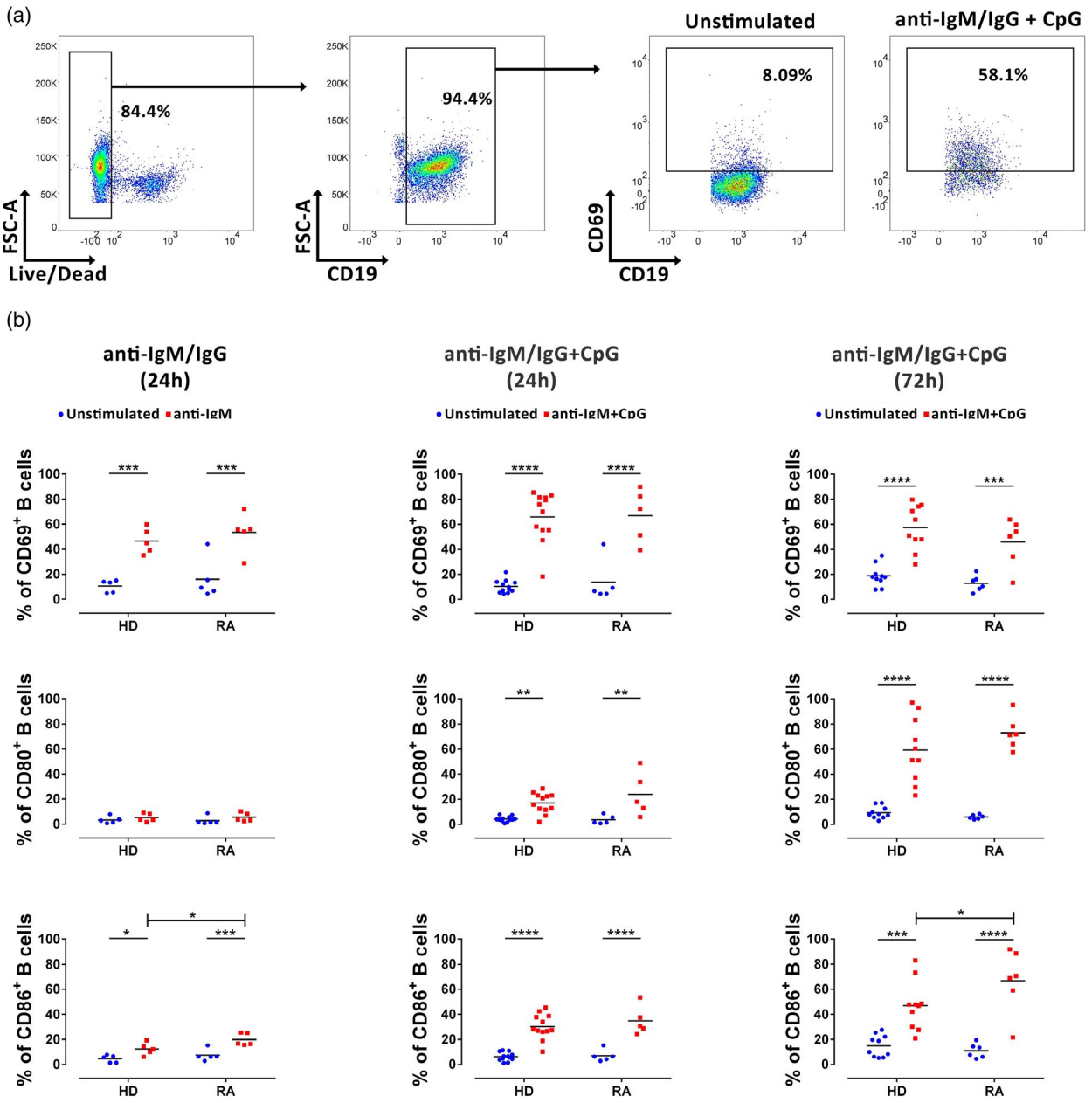

Fig. 6. Increased expression of CD86 in B cells from patients with seropositive rheumatoid arthritis (RA) compared with healthy donors (HD). (a) Representative pseudocolor plots of enriched and cultured B cells from HD, analyzed according to Live/Dead and CD19 expression. The frequency of positive B cells to CD69 on unstimulated and anti-immunoglobulin (Ig)M/IgG plus cytosine–phosphate–guanine oligodeoxynucleotide (CpG) stimulated cells for 72 h is shown. (b) Mean and frequency data of B cells positive to activation markers (CD69, CD80 and CD86) in patients with seropositive RA and HD after the following stimuli: anti-IgM/IgG for 24 h (left, RA $n = 5$, HD $n = 5$), anti-IgM/IgG plus CpG for 24 h (center, RA $n = 5$, HD $n = 12$) and 72 h (right, RA $n = 6$, HD $n = 10$). Two-way analysis of variance (ANOVA) test with Šidák post-test, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, $^{****}P < 0{\cdot}0001$.

of patients with seropositive, but not seronegative RA. It has been reported that CD21^{-} cells from HD express activation markers such as CD86 and CD95 [46,47], suggesting that these cells have been previously activated *in vivo* and can act as antigen-presenting cells. Other investigators have proposed that CD21^{-} cells from the tissues of patients with RA resemble exhausted B cells. Interestingly, CD21^{-} B cells have been detected in synovial fluid from patients with seropositive RA. CD21^{-} B cells expressing the CD27^{-}IgD^{-} phenotype represents the population with the highest frequency of RANKL^{+} cells [48]. Furthermore, it has reported that the

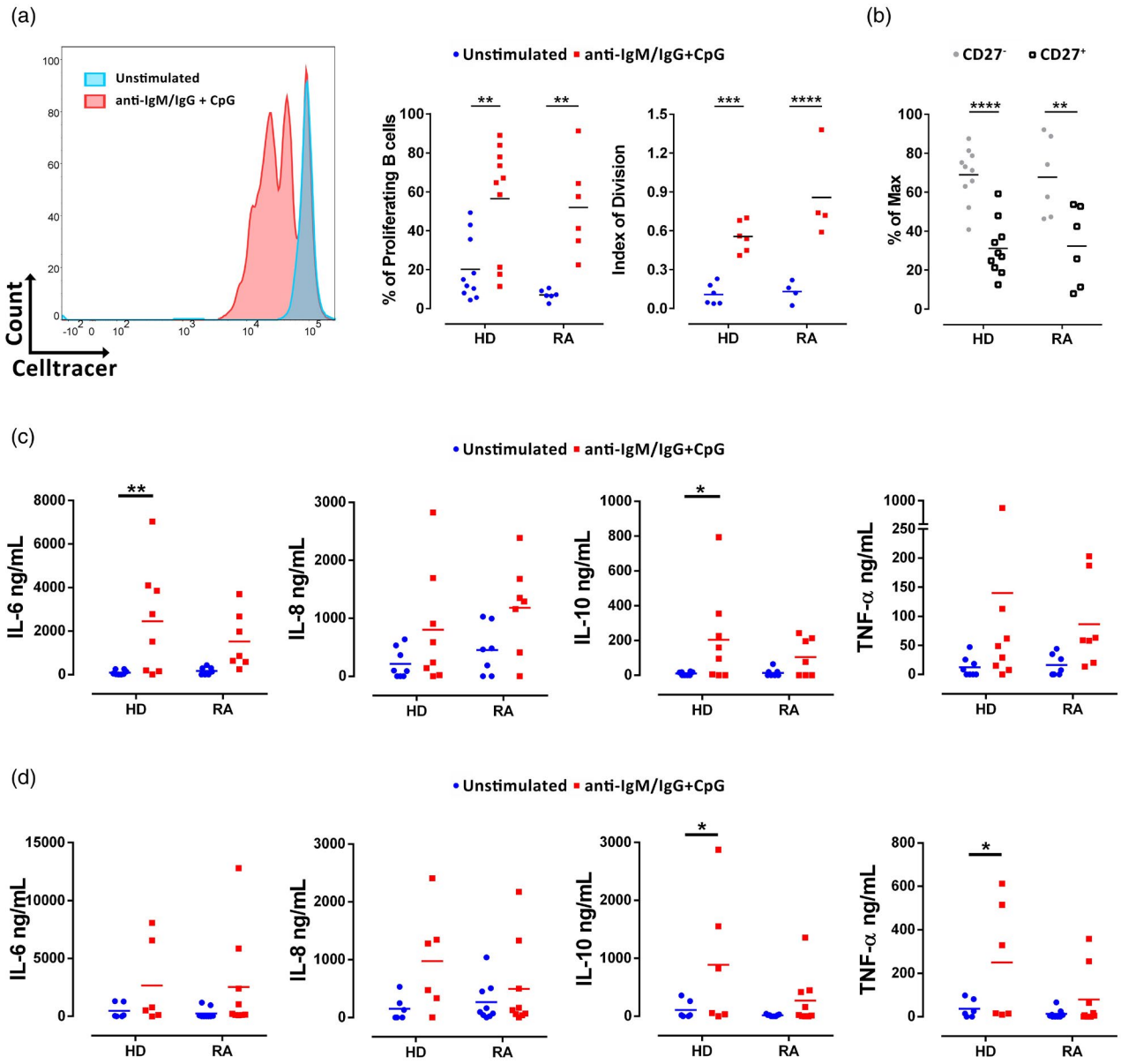

Fig. 7. No significant increase in interleukin (IL)-6 and IL-10 levels by activated B cells from patients with seropositive rheumatoid arthritis (RA). (a) Representative histograms of B cell proliferation (left) in unstimulated (blue) and anti-immunoglobulin (Ig)M/IgG + CpG stimulated (red) B cells from a healthy donor (HD) (72 h). Mean and frequency data of divided B cells from six patients with RA and 10 HD (center). Comparison of the index of division between six patients with RA and seven HD is shown (right). (b) Frequency of $CD27^+$ and $CD27^-$ cells in proliferating B cells from HD and patients with seropositive RA after culture of 72 h with anti-IgM/IgG + CpG. Median and frequencies from 10 HD and six patients with seropositive RA are shown. (c,d) Production of IL-6, IL-8, IL-10 and tumor necrosis factor (TNF)-α in HD ($n = 6$) and patients with seropositive RA ($n = 9$) after anti-IgM/IgG plus CpG for (c), 24 h and (d), 72 h. Two-way analysis of variance (ANOVA) test with Šidák post-test, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

$CD21^-$ B cell population is expanded and displays characteristics of exhausted memory B cells during chronic inflammatory conditions, such as viral infections including HIV and hepatitis C [49,50]. This suggests that $CD21^-$ cells are generated from long-term contact with specific antigens. Therefore, both of these atypical B cell populations, $CD27^-IgM^-IgD^-$ and $CD21^-$ [37], appear to correspond with memory B cells that have been chronically activated and expanded in patients with seropositive RA, but not in seronegative patients. The $CD21^-CD38^{low/-}CD24^-$ and $CD21^-CD38^{low/-}CD24^+$ populations (even in HD) have shown higher numbers of cell divisions than their counterparts in $CD21^+$ B cells [51]. This characteristic, the increased mortality of B

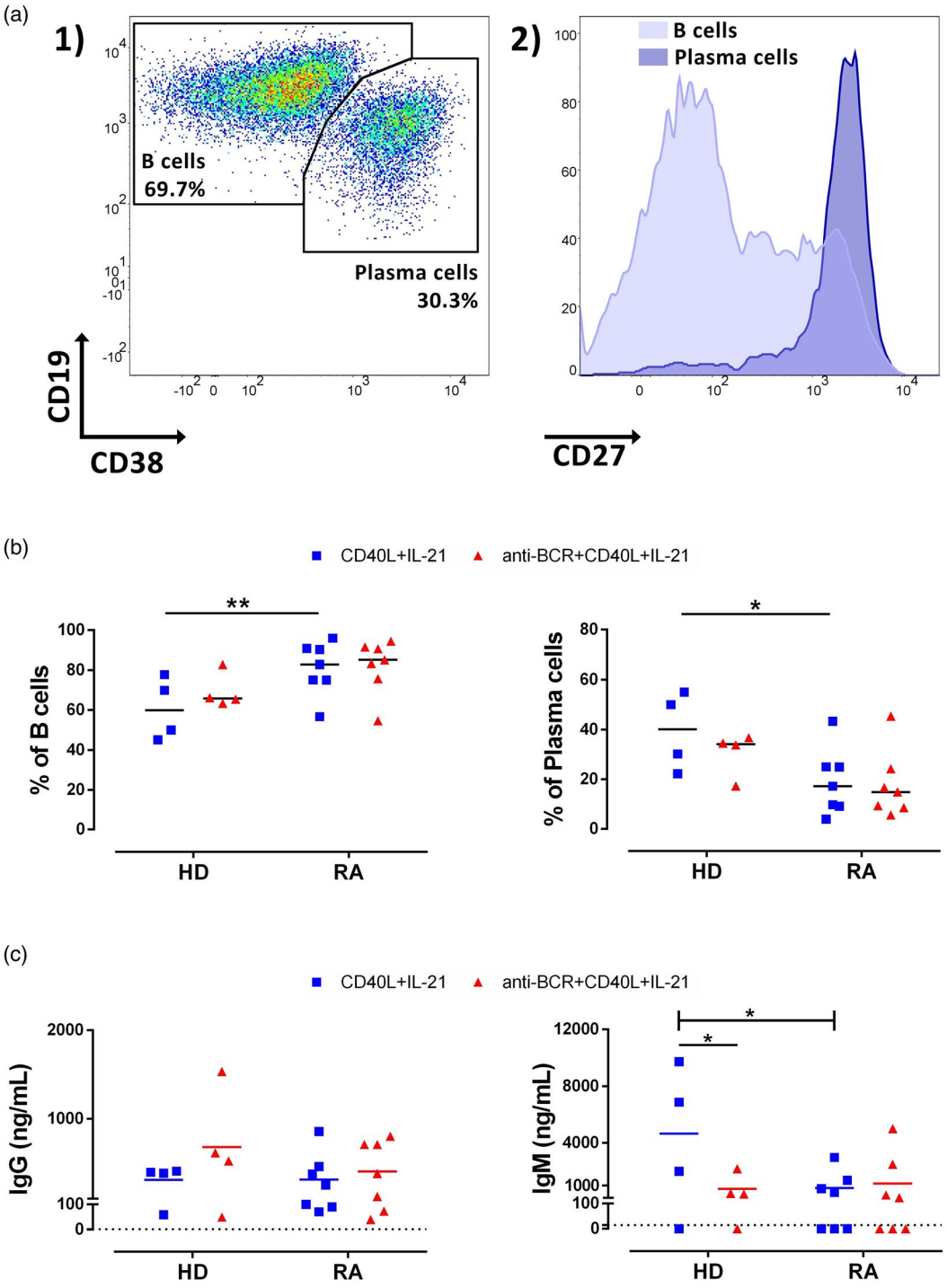

Fig. 8. Low *in-vitro* generation of plasma cells in patients with seropositive rheumatoid arthritis (RA). Enriched B cells were cultured for 7 days with CD40L + IL-21 in the presence or absence of anti-immunoglobulin (Ig)IM/IgG, as described in the Materials and methods section. (a) (1) Representative analysis of plasma cells and other B cells from a healthy donor (HD) after culture. Viable cells (Live/Dead^{-}) were divided into B cells ($CD19^{+}CD38^{low/-}$) and plasma cells ($CD19^{low/-}$ + $CD38^{+}$). (2) Representative histogram of CD27 expression in other B cell and plasma cell populations from HD. (b) Mean and frequency of B cells and plasma cells according to (a). (c) Mean and levels of total IgG and IgM production in cultures of enriched B cells from HD and patients. Dotted line indicates the reported sensibility of enzyme-linked immunosorbent assay (ELISA); four HD and seven RA patients are shown. Two-way analysis of variance (ANOVA) test with Šidák post-test, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

cells lacking CD21 expression [52] and the CD21 role in germinal center reaction [53], suggest a potential extra-follicular or germinal center-independent activation and differentiation of these B cells in seropositive patients.

We observed that a significant fraction of the $CD27^{-}IgM^{-}IgD^{-}$ cells are also $CD21^{-}$. Previously, a subpopulation of double-negative B cells that do not express CXCR5 (known as DN2) was reported increase in SLE patients and was identified as precursor of autoantibody-producing plasma cells [54]. Thus, it is possible that the presence of these cells in seropositive RA is not only the result of chronic antigen exposure, but may also contribute to the immunopathology as a source of ACPA and as antigen-presenting cells. In fact, the ratio of DN2 : DN1 B cells was increased in both SLE and COVID-19 patients and was associated with increased extra-follicular B cell responses [55]. Interestingly, COVID-19 patients exhibited a characteristic profile of increased frequency of B cells with $CD21^{-}$ and $CD27^{-}IgM^{-}IgD^{-}$ phenotypes [55]. Nevertheless, more studies will be required to establish the mechanisms through which $CD27^{-}IgM^{-}IgD^{-}$ and $CD21^{-}$ B cells are generated and their pathological role in seropositive RA.

We found decreased expression of CD22 in patients with both seronegative and seropositive RA compared with HD. Decreased CD22 expression has been previously reported in patients with systemic sclerosis [27] and SLE [26]. Also, it has been reported that patients with RA have a lower frequency of $CD22^{+}$ B cells compared with HD [56]. It has been observed in mice that CD22 expression is down-regulated following BCR stimulation, but not after LPS or CD40L treatment [57]. This suggests that CD22 expression on B cells from patients with RA is reduced because of chronic antigen stimulation. This idea is also reinforced by the fact that the reduction in CD22 was observed mainly in memory subsets ($CD27^{+}IgM^{+}$, $CD27^{+}IgM^{-}$ and $CD27^{-}IgM^{-}$ cells). The lower expression of the regulatory molecule CD22 could facilitate the activation of autoreactive B cells and contribute to the progression of the disease, as suggested in $CD22^{-/-}$ mice that show an increase in the frequency of B cells with a germinal center phenotype in the absence of specific immunization [58]. We propose that this decrease in CD22 expression could partially explain the increased frequency of $CD86^{+}$ cells found in B cells from patients with seropositive RA compared with HD. The high expression of CD86 in B cells from patients with seropositive RA may also enhance the ability of these cells to co-stimulate and activate CD4 T cells [59]. However, further studies are required to test this hypothesis. In addition, patients with RA may be unresponsive to emerging biological therapies targeting CD22 such as epratuzumab [28], because of the low expression of this molecule on B cells.

We observed that B cells from seropositive patients were not efficient at producing cytokines, in particular IL-10, after anti-BCR plus CpG stimulation compared with B cells from HD. The defective IL-10 secretion observed in seropositive patients may be related to a previous report that indicated a lower frequency of regulatory B cells (B10, B cells IL-10^{+}) in RA patients compared with HD [60]. Reduced *in-vitro* cytokine production by B cells has been previously reported in patients with SLE, especially in those with high disease activity [61–63]. Reduced regulatory action of B cells in RA patients could influence inflammation, autoantibody production and interactions with other cells, such as T cells.

As mentioned previously, a possible scenario that may explain the alterations in phenotype and function observed in B cells from seropositive patients is an excess of stimulation *in vivo* resulting in a post-activated state or exhausted B cells. They may be considered as hyporesponsive B cells induced by chronic activation/stimulation by specific antigens [64,65]. Interestingly, patients with common variable immunodeficiency that develop autoimmune manifestations also present with a high frequency of $CD21^{-}$ B cells concomitant with an increased frequency of exhausted programmed cell death 1 (PD-$1)^{+}CD4^{+}$ T cells [66]. In autoimmune diseases such as SLE, weak cytokine production by B cells has been associated with previous *in-vivo* stimulation with circulating CpG [61] and the presence of an exhausted or post-activated status [64]. A similar scenario could be occurring in patients with seropositive RA because of an excess of citrullinated peptides in circulation and synovial fluid that can bind TLRs [67] and the BCR, which leads to chronic B cell activation.

Another potential explanation for the low response of B cells is the presence of anergic B cells. This has been reported in mouse models of autoimmunity exposed to chronic antigen stimulation lacking a proper T cell signal. Anergic B cells are characterized by defects in calcium mobilization and tyrosine phosphorylation [68]. In autoimmune patients with Sjögren syndrome, RA or SLE, augmented frequency of $CD21^{-}$ B cells have been related to anergy [40,69,70]. However, we did not observe differences in the frequency of B cells with the $CD27^{-}IgM^{-}IgD^{+}$ anergic phenotype. Neither disparities in calcium mobilization nor global phosphorylation of tyrosine occurred in subsets with anergic phenotype between patients with seropositive RA and HD. Therefore, our data suggest that alteration in B cells of seropositive RA patients does not appear to be related with early signaling defects or a higher frequency of anergic B cells. Instead, it is more likely that an expansion of chronically activated cells and memory populations occurs, which are hyporesponsive because of a persistent antigenic exposure (exhausted).

We observed a decrease in the *in-vitro* generation of plasma cells and a lower production of IgM in cultures of B cells from seropositive patients compared with HD. We cannot rule out that the lack of differences in IgG and the decrease in IgM may be explained by other isotype changes, such as IgA [71]. However, it is worth noting that anti-IgM/IgG treatment result in IgM production and plasma cell differentiation of HD to comparable levels with that observed in seropositive patients without anti-BCR. This suggests that previous antigen recognition of B cells in these patients could impact *in-vitro* differentiation and humoral responses. It is also possible that a decrease in differentiation to plasma cells is explained by a low autocrine production of IL-10 by B cells from patients, which is an important cytokine for plasma cell development [72]. As B cells from patients with seropositive RA exhibited a high frequency of $CD86^+$ cells and low differentiation of plasma cells, these B cells may act as co-stimulatory cells toward other key participants in RA, such as CD4 T cells [73].

The interpretation of our results may be biased because of the *in-vitro* treatments used, and there is no clear information as to how $CD21^-$ and $CD27^-IgM^-IgD^-$ B cells respond to *in-vitro* stimulation, or whether these cells are activated and differentiated by conventional routes. Another limitation in the present work is the lack of functional characterization of sorted $CD21^-$ and $CD27^-IgM^-IgD^-$ B cell populations. Our results encourage the necessity to perform further studies in these B cell subsets for a clearer understanding of their biology and involvement in RA. Also, we cannot overlook the effect of medication in our results. A significant increase in the frequency of $CD27^-IgM^-IgD^-$ B cells in patients with seropositive RA with Leflunomide treatment was observed compared to patients with seropositive RA without this drug (Supporting information, Fig. S6). Previously, it has been reported that patients with RA treated with Leflunomide showed reduction in frequency and number of memory B cells [74]. We did not observe changes in frequency of other memory subsets, either in the frequency of $CD21^-$ B cells or the expression of CD22 on B cells according to the other drug treatments (Supporting information, Fig. S6 and data not shown). To definitively rule out the effect of the therapy, further studies that evaluate atypical B cell subsets and their responses in patients with seropositive and seronegative RA over time, before and after treatment, and according to the activity of the disease would be desired.

In general, the alterations observed in B cells from patients with seropositive RA appear to be more related to changes in activated, rather than anergic cells. Previously, we have reported that patients with seropositive RA had increased serum levels of cytokines (e.g. IL-6, IL-1β and TNF-α) compared with seronegative patients and HD [7]. These soluble factors may contribute to the reported differentiation of peripheral helper T (T_{PH}) cells in the synovia of RA patients [75], cells associated with B cell activation and antibody production in ectopic lymphoid structures. This extra-follicular or germinal center-independent activation of B cells may result in an increase of $CD21^-$ and $CD27^-IgM^-IgD^-$ B cells [37,55]. The presence of these atypical B cells in the circulation may be the reflection of B cells recruited to joint tissue that become exhausted after chronic exposition to antigens [48], and therefore could be important contributors of tissue inflammation in patients with seropositive RA. However, more studies are required to determine the contribution of these atypical $CD21^-$ and $CD27^-IgM^-IgD^-$ B cell populations to the development and pathology of RA.

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Disclosures

None of the authors have financial or commercial conflicts of interest.

Author contributions

H. R.-A. performed the experiments and analyzed the results. A. V.-G., C. M.-V. and G.V. enrolled RA patients. J. O.-E. collected and managed the clinical data of patients. H. R.-A. and D. C. drafted the manuscript. M. R. and G. V. critically revised the manuscript. H. R.-A., M. R., G. V. and D. C. contributed to the study design. D. C. directed the study.

Data Availability Statement

All data generated during this study are available from the corresponding author on reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Fig. S1. No differences in the numbers of B cell subsets between patients with RA and HD. A. Comparison of the absolute counts of transitional, plasma cell, $CD27^-$, and $CD27^+$ subsets from HD and patients with seronegative ($CCP3^-$) and seropositive ($CCP3^+$) RA. B. Number of $CD38^{low/-}$ cells according to CD27 and IgM expression from HD and patients with $CCP3^-$ and $CCP3^+$ RA. Data and median of 12 HD, 6 patients with $CCP3^-$ RA and 17 patients with $CCP3^+$ RA are shown. Kruskal–Wallis test and Dunn's post-test.

Fig. S2. No differences in frequency of B cell subsets in patients with active RA and in remission. Median and frequency of each B cell subset according to the analysis shown in Fig. 2A among patients with active RA (DAS28 >2.6, aRA), patients in remission (DAS28 ≤2.6, rRA), and HD; 12 HD, 12 rRA patients and 11 aRA patients are shown. Kruskal–Wallis test and Dunn's post-test.

Fig. S3. No differences in FcμR and FcγRIIb expression between HD and patients with RA. (a) Representative histograms of A. FcμR, (b) FcγRIIb and (c). CD22 expression on B cells from HD. (d–e) Comparison of the MFI of FcγRIIb between HD and patients with RA according to D. CCP3 seropositivity and E. DAS28 score. (f–g) Comparison of the MFI of FcμR between HD and patients with RA according to F. CCP3 seropositivity and G. DAS28 score. Data and median of 11 HD, 6 patients with $CCP3^-$ RA, 17 patients with $CCP3^+$ RA, 12 rRA patients and 11 aRA patients are shown. Kruskal–Wallis test, Dunn's post-test.

Fig. S4. No differences in calcium mobilization of $CD27^-CD21^-$ and $CD27^-IgM^-IgD^+$ B cells between HD and patients with seropositive RA. Kinetic of Indo 1AM ratio in unstimulated B cells for 30 s (baseline) and stimulated B cells with anti-IgM/IgG for 90 s of A. $CD21^-CD27^-$ and B. $CD27^-IgM^-IgD^+$ B cells from patients with seropositive RA and HD. Mean and the standard error of the mean of 5 HD and 5 patients with RA are shown. Two-way ANOVA test with Šidák post-test.

Fig. S5. No differences in frequency of memory subsets between B cells from patients with seropositive RA and HD after culture. Mean and frequency of B cell subsets according to CD27 and IgM expression in B cells after 7 days of culture. Data from 4 HD and 7 patients with seropositive RA are shown. Two-way ANOVA test with Šidák post-test.

Fig. S6. Effect of treatment in atypical B cell populations and CD22 expression. (a-b) Frequency of A. $CD27^-IgM^-IgD^-$ and B. $CD21^-$ B cells in patients with seropositive RA with or without Methotrexate (MTX, left), Prednisone (PDN, center) and Leflunomide (Left, right) treatments. © MFI of CD22 on total B cells of patients with seropositive RA, classified according to drug treatment as described in A-B. Data and median of 17 patients with CCP3+ RA are shown. Mann Whitney test. $*P < 0{\cdot}05$.